Protein expression and subcellular localization of familial acute myelogenous leukemia-related factor

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Abstract. The present study was designed to evaluate the expression and subcellular distribution of the familial acute myelogenous leukemia-related factor (FAMLF). A 14-amino acid epitope of the predicted open reading frame of the *FAMLF* gene was identified using bioinformatics. This polypeptide was synthesized, conjugated to keyhole limpet hemocyanin and was subsequently used to produce antibodies. The antibody titer and specificity were characterized using ELISA and western blot assays, respectively. The antibody detected FAMLF protein expression in several human leukemia cell lines, bone marrow cells derived from one acute myeloid leukemia patient and one chronic myeloid leukemia patient, but not in bone marrow cells of healthy subjects. The FAMLF/GFP fusion protein was expressed in both the nucleus and the cytoplasm of transfected NIH3T3 cells. Our results demonstrate that the *FAMLF* gene is expressed in an AML patient but not in healthy controls, suggesting its association with AML.

Introduction

Acute myeloid leukemia (AML) is the most common hematological malignancy in adults, with a worldwide incidence of ~3.6 cases in 100,000 individuals (National Cancer Institute. SEER Stat Fact Sheets: Acute Myeloid Leukemia; http://seer.cancer.gov/statfacts/html/amyl.html). A number of AML-related chromosomal abnormalities and gene mutations have been identified, and their involvement in the regulation of DNA methylation, signal transduction, energy synthesis, and cellular structure has been reported (1).

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Abbreviations: AML, acute myeloid leukemia; FAMLF, familial acute myelogenous leukemia-related factor

Key words: familial acute myeloid leukemia, *FAMLF*, antibody, nuclear expression

The identification of new AML-related genes may provide insight into disease prognosis, treatment response, and new therapeutic regimens for patients with AML. Mutations in CCAAT enhancer binding factor α (*CEBPA*) have been associated with prolonged recurrence-free survival in AML patients (2). Mutations in nucleophosmin-1 (*NPM1*) and the internal tandem duplications in *FLT3* predict positive AML prognosis (3), and mutations in several genes such as *MLL-PTD, FLT3/ITD, WT1,* and *C-KIT* predict negative AML prognosis (4-7). Thus, screening for mutations in *CEBPA, NPM1, FLT3,* and *KIT* is part of the routine workup for AML (8,9).

A number of drugs targeting AML-related genes have been tested for their efficacy in treating AML, including TNF-related apoptosis inducing ligand (TRAIL) and tipifarnib, an inhibitor of farnesyltransferase (FTase), alone or in combination with other anti-leukemia drugs (10,11). Inhibitors of histone deacetylase, proteasome and Bcl-2 antisense oligonucleotides have also been used as targeted drugs in the clinical treatment of AML (12). Furthermore, inhibitors of Fms-like tyrosine kinase 2 (FLT-2), such as lestaurtinib (CEP701), tandutinib (MLN518) and PKC412 have shown benefit in treating FLT3-associated AML (13).

Previously, we identified a large family with a high incidence of AML in Fujian China (14). A total of 11 family members in 4 generations were diagnosed with AML; two patients developed acute erythroleukemia (M6), one suffered from minimally differentiated acute myeloblastic leukemia (M1), one had AML, one had acute monocytic leukemia (M5), and the remaining 6 were diagnosed with acute myeloid leukemia with partial differentiation (M2).

Using subtractive hybridization and rapid-amplification of cDNA ends (RACE) techniques, the bone marrow biopsy of an 11-year-old boy with M2 from the above family and the expressed sequence tag (EST) zywb4 were used to clone the full-length cDNA of a novel familial acute myelogenous leukemia-associated gene (15). A sequence was cloned, mapped to human chromosome 4, and contained one open reading frame of 2257 bp coding for a sequence of 144 amino acids and a poly(A) tail. The newly predicted gene was designated as ELF2C (GenBank accession no. DQ359746).

In addition, another EST sequence zywb87 was also used to clone a familial acute myelogenous leukemia-associated gene (16). A full-length cDNA of 2313 bp with a complete

open reading frame (ORF) of 249 bp was obtained from the bone marrow specimen of the above-mentioned familial AML male patient. Localized on human chromosome 1q31.3, the gene coded an 82-amino acid polypeptide with a leucine-rich repeat and was named familial acute myelogenous leukemia-related factor (FAMLF; GenBank accession no. EF413001; protein accession no. ABN58747). The *FAMLF* gene expression level was significantly higher in AML patients outside this family than in normal healthy persons outside this family (16).

In the present study, the polyclonal antibody to FAMLF was generated and used to assess the protein expression of FAMLF in bone marrow cells collected from an AML patient and a chronic myeloid leukemia (CML) patient, as well as in several human cell lines. In addition, cell lines were transfected with the FAMLF/GFP fusion protein expression vector to investigate the subcellular localization of the FAMLF protein.

Materials and methods

The present study was approved by the Ethics and Institutional Animal Care and Use Committees of the Fujian Medical University Union Hospital.

Preparation of a polyclonal antibody against FAMLF. The hydrophilicity and antigenicity of the 14-amino acid N-terminal polypeptide of the FAMLF protein (amino acids 3-16) were predicted using MacVector (IBI Ltd., Cambridge, UK), and it was synthesized by Shanghai Chaobao Biotech (Shanghai, China). The polypeptide was subsequently purified by high pressure liquid chromatography and mass spectrometry; the purity of the resulting polypeptide was as high as 92%. The purified polypeptide was conjugated to keyhole limpet hemocyanin (KLH) protein (Shanghai Chaobao Biotech). The resulting polypeptide-KLH was used for immunization.

Preimmunization venous blood (1-2 ml) collected from the ear vein of 2 healthy male New Zealand white rabbits 7 days before immunization was used as a negative control. Then, equal volumes of 200 μg of polypeptide-KLH complex and complete Freund's adjuvant (CFA) were mixed and emulsified. This emulsion was subcutaneously injected at multiple sites along the back. The animals were immunized once per week with CFA being replaced with incomplete Freund's adjuvant (IFA) on alternate weeks.

At week 7, blood samples were collected from the ear vein daily, and the IgG titer was then quantified by enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000; Pierce Thermo Scientific, Rockford, IL USA) according to the manufacturer's instructions. When the IgG titer was higher than 10,000, the anti-FAMLF antibody in the collected serum was purified using an IgG affinity column (Pierce Thermo Scientific) according to the manufacturer's instructions.

Western blot assay. Antibody specificity was examined using western blot analysis. Total protein lysates were prepared from bone marrow cells derived from an AML patient and a CML patient, NIH3T3 cells expressing the FAMLF-GFP fusion protein (described below), and several human cell lines, including the COLO205 human colon adenocarcinoma cell line; the NB4 human acute promyelocytic leukemia cell line; the U937 human macrophage-like cell line; the k562 human myeloid leukemia cell line; the U266 human myeloma cell line; the HL60 human promyelocytic cell line; the CA46 human lymphoma cell line; and the CEM human T cell lymphoblast-like cell line (Institute of Hematologic Diseases, Fujian, China). Total protein lysates (40 μg/lane) were separated by Tricine SDS-PAGE. Blots were incubated with the primary anti-FAMLF antibody (1:250) or the anti-GFP antibody (1:1,000), and subsequently with HRP-conjugated goat anti-rabbit IgG (1:4,000; Pierce Thermo Scientific). Protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham, Little Chalfont, UK).

PCR amplification of FAMLF DNA. Total RNA was isolated from U937 cells, a human macrophage-like cell line, using TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA samples were reverse transcribed by MultiScribe™ reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The sequences of the FAMLF forward and reverse primers (which were designed to include the *Bgl*II and *Sal*I restriction sites but not the stop codon) were: forward, 5'-GGAAGATCTAT GAAAACAGGGATTC-3' and reverse, 5'-ACGCGTCGAC TTGATTGTTGGTAAT-3'. The above cDNA samples along with FAMLF PCR primers were amplified by *Taq* DNA polymerase (Invitrogen). The PCR conditions for FAMLF amplification were as follows: 94˚C for 5 min, followed by 35 cycles of 94˚C for 45 sec, 54˚C for 30 sec, and 72˚C for 30 sec and a final extension at 72˚C for 10 min. The products were purified using agarose gel electrophoresis and subsequently sequenced.

Construction of the eukaryotic expression vector expressing GFP/FAMLF. The purified PCR product was digested with *Bgl*II and *Sal*I, and then was ligated by T4 DNA ligase (Invitrogen) with the vector pAcGFP-N1 (Clontech, Mountain View, CA, USA) that was digested with the same enzymes. Consequently, the *FAMLF* coding region was inserted upstream of *GFP*. The sequence of the *FAMLF/GFP* was verified using DNA sequencing. The resulting FAMLF-containing pAcGFP-N1 vector was used to transform the competent TOP-10 cells (Invitrogen), which were then plated on kanamycin agar medium. The positive colonies were selected, and the recombinant plasmid DNA was subsequently extracted and sequenced to confirm the existence of the *FAMLF* gene sequence.

FAMLF subcellular localization as determined using the FAMLF/GFP fusion protein. NIH3T3 cells were maintained in RPMI-1640 medium containing 10% newborn calf serum and incubated with 5% CO_2 at 37˚C for 24 h. The *FAMLF/GFP* plasmid was transfected into NIH3T3 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, cultured for an additional 24 h, fixed with 4% formaldehyde and viewed under a laser scanning confocal microscope (LSCM). Since the expression of *FAMLF* was driven by the *GFP* promoter in the *FAMLF/GFP* fusion gene, green fluorescence was able to be used as a marker for the expression and localization of the FAMLF protein.
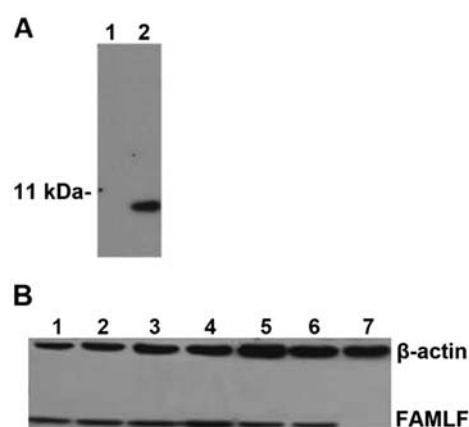
Figure 1. Detection of FAMLF protein expression in several human cell lines. (A) Western blot analysis of the FAMLF protein in bone marrow cells of a healthy control (lane 1) or in COLO205 colon adenocarcinoma cells (lane 2) using a polyclonal antibody to the FAMLF protein. The location of 11 kDa is indicated by a molecular marker. (B) Western blot analysis detected the FAMLF protein in NB4 acute promyelocytic leukemia cells (lane 1), U937 macrophage-like cells (lane 2), k562 myeloid leukemia cells (lane 3), U266 myeloma cells (lane 4), HL60 promyelocytic cells (lane 5), CA46 lymphoma cells (lane 6), but not in CEM T cell lymphoblast-like cells (lane 7).
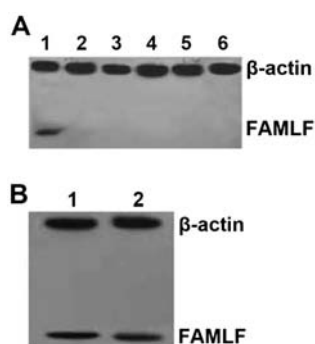

Figure 2. Detection of FAMLF protein expression in AML and CML patients by western blot analysis. (A) FAMLF protein was detected in the bone marrow cells of one AML patient (lane 1), but not in the bone marrow cells of the healthy controls (lanes 2-6). (B) FAMLF protein was detected in the CA46 lymphoma cell line (lane 1) and in bone marrow cells of one CML patient (lane 2).

Results

Antibodies were constructed against a 14-amino acid sequence located at the N-terminus of the predicted open reading frame of the *FAMLF* gene. ELISA detected specific antibody in the blood collected after but not before immunization (data not shown). Western blot analysis using the purified anti-FAMLF polyclonal antibody identified an 8-kDa protein (consistent with the predicted size of the FAMLF protein) in the COLO205 colon adenocarcinoma cells but not in the bone marrow cells of a healthy control (Fig. 1A) and detected the FAMLF protein in NB4 acute promyelocytic leukemia cells, U937 macrophage-like cells, k562 myeloid leukemia cells, U266 myeloma cells, HL60 promyelocytic cells, CA46 lymphoma cells, but not in CCRF CEM (T-cell lymphoblast-like) cells (Fig. 1B).

Western blot analysis further detected FAMLF protein expression in the bone marrow cells from one AML patient
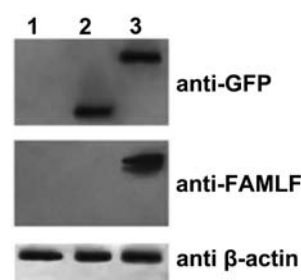

Figure 3. Validation of the FAMLF/GFP fusion protein construct. NIH3T3 cells (lane 1), NIH3T3 cells transfected with pAcGFP-N1 vector (lane 2), and NIH3T3 cells transfected with a plasmid that expressed the 35-kDa FAMLF/GFP fusion protein (lane 3). GFP protein (top panel), FAMLF protein (middle panel) and β-actin were detected by western blot analysis.
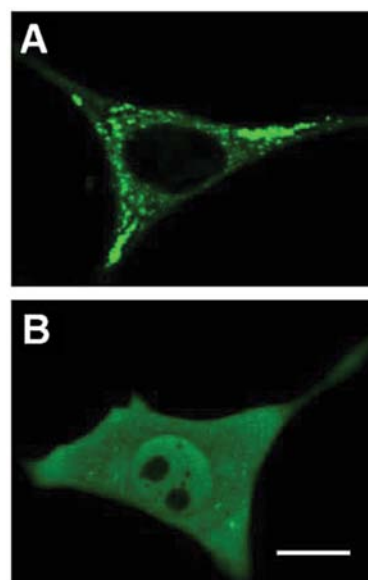

Figure 4. Subcellular localization of the FAMLF protein. Confocal microscopy was used to examine NIH3T3 cells transfected with (A) the pAcGFP-N1 expression vector or (B) the FAMLF/GFP expression vector. Scale bar, 5 μm.

(Fig. 2A, lane 1) and one CML patient (Fig. 2B, lane 2) but not in the healthy controls (Fig. 2A, lanes 2-6).

Western blot assay of FAMLF/GFP fusion protein expression also cross-validated the specificity of the FAMLF antibody. The anti-GFP antibody identified a 27- and 35-kDa protein in the NIH3T3 cells transfected with vectors expressing GFP or the FAMLF-GFP fusion protein, respectively (Fig. 3; top panel). The anti-FAMLF antibody also detected a 35-kDa protein from the same protein extracts (Fig. 3; middle panel), suggesting that both anti-GFP and anti-FAMLF antibodies specifically recognized the FAMLF/GFP fusion protein.

Furthermore, confocal microscopy of NIH3T3 cells revealed localization of GFP alone only in the cytoplasm (Fig. 4A) and the FAMLF/GFP fusion protein in both the nucleus and the cytoplasm (Fig. 4B).

Discussion

The FAMLF antibody detected a single protein in the extract of bone marrow cells from one AML patient and one CML

patient, but not from the healthy controls. The size of the detected protein (8 kDa) was consistent with the predicted molecular weight of the FAMLF protein. FAMLF/GFP fusion protein was found in the nucleus and cytoplasm. Our results indicate for the first time that the FAMLF protein is associated with AML and may participate in activities within the nucleus.

Prior to the present study, no antibody against FAMLF had been developed. In the present study, we used a previously described strategy for generating antibodies against particular proteins (3,4,6). This approach requires the selection of a specific peptide epitope. In general, to enhance antigen immunoreactivity, the peptide should have strong hydrophilicity and a stable conformation, and either end of the peptide should be linear to maximize its availability for antibody binding (5,7). Using this criterion, we selected a 14-amino acid sequence (CALWVSGIFVDEVI) at the N-terminus of the FAMLF protein that was predicted to have high hydrophilicity, be readily accessible to antibody, and be specific to FAMLF, thereby reducing the likelihood of non-specific binding.

We also maximized the likelihood of producing high quality antibodies by careful selection of animals, preparation of the adjuvant, selection of the antigen dose injected and maximization of immunogenicity. In regards to maximizing the immunogenicity of the antigen, the synthesized peptide was purified to ~92% purity, conjugated to KLH, and emulsified in adjuvant (CFA and IFA). The IgG titer was monitored using ELISA until the titer was higher than 1:10,000, and the specificity of the FAMLF antibody was confirmed using western blot analysis.

Families with familial AML are powerful resources for the identification of possible underlying genetic determinants of AML. Many families who carry mutations in the *GATA2*, *TERT*, *TERC*, *RUNX1* and *CEBPA* genes have been repeatedly identified (17-22). In contrast, the *FAMLF* gene identified in the Fujian AML family (14) was expressed only in affected individuals but not in healthy controls, suggesting that this gene has a distinct pathophysiological role.

A relatively large number of genes with diverse functions have been associated with AML (1-7), suggesting distinct underlying mechanisms of AML. The biological function of *FAMLF* and its pathophysiological role in AML are still unclear. In the present study, the expression of FAMLF was evaluated in only one Fujian family member who had AML. It will be of interest to determine how penetrant this expression phenotype is in family members of the Fujian family and other familial and sporadic AML patients and whether other mutations within the *FAMLF* gene, including regulatory regions, are associated with AML in the Fujian family.

Our findings suggest that the FAMLF protein is a relatively small protein (8 kDa), may translocate to the nucleus or be associated with the nuclear membrane, and may act as a transcription factor to modulate gene expression. The FAMLF/GFP protein was also found in the cytoplasm, suggesting that the FAMLF protein may be shuttled between the nucleus and cytoplasm similar to a transcription factor. Additional research is warranted to investigate the subcellular localization and biological function of the FAMLF protein.

In conclusion, we generated an antibody to the predicted protein of the *FAMLF* gene and showed, for the first time, that the FAMLF protein was expressed in several leukemia cell lines and in the bone marrow cells of an AML patient and a CML patient. This protein was present in both the nucleus and the cytosol.

Acknowledgements

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